2 MATERIALS

2.1 Devices

Blotting apparatus:	Transblot® SD Semi-Dry, Biorad
Centrifuges:	Biofuge fresco, Kendro
	Megafuge 1.0R, Heraeus
	RC 5C Plus, Sorvall
	L8-M Ultracentrifuge, Beckman
FACS:	FACSCalibur™ BD, Biosciences
Freezing container:	Qualifreeze, Qualilab
Gamma counter:	Perkin-Elmer (Packard) Cobra II Gamma Counter
Gel dryer:	Model 583, Biorad
Gel electrophoresis apparatus:	Agagel mini, Biometra
	Agagel Maxi, Biometra
	mini-protean® 3 Cell, Biorad
	MAXI-Vertikal, Roth
Gradient former:	Model 150, Gibco
Microscope:	Axiovert 25, Zeiss
Thermocycler:	GeneAmp® PCR System 2400, Perkin Elmer
Power supplies:	Power Pac 300, Biorad
	EPS 601, Amersham Pharmacia biotech
Refrigerating circulator:	Ecoline RE 104, Lauda
Scales:	BP 210 S, Sartorius
Sequencer:	ABI PRISM™ 377 DNA Sequencer, Applied Biosystems
Shakers:	3005-3020, GFL
	REAX 2, Heidolph
	Thermomixer comfort, Eppendorf
Sonicator:	Branson Sonifier 450
UV-transilluminator:	TFP-M/WL, Vilber Lourmat

2.2 Biochemicals and chemicals

Chemicals not separately listed were obtained from the company Roth.

Amersham Pharmacia Biotech:	Con A Sepharose; Protein A Sepharose CL-4B; Protein G Sepharose 4 fast flow; [^{14}C] Protein molecular weight marker; Redivue PRO-MIX L-[^{35}S] in vitro Cell Labelling Mix (L-methionine/L-cysteine)
Biorad:	Ethidiumbromide; Kaleidoscope Prestained Standards
BioWhittaker Molecular Applications:	RPMI 1640 without L-Cysteine, L-Methionine; SeaKem® LE Agarose
Calbiochem:	Digitonin, high purity; iodoacetamide (IAA)
Fermentas:	Calf intestinal alkaline phosphatase; T4 DNA ligase
Gibco/Invitrogen:	Dulbeccos Modified Eagle Medium (DMEM) pulver, high glucose, pH 7,2; fetal calf serum (FCS); penicillin (10.000U/ml)/Streptomycin (10.000µg/ml); L-glutamin (200mM; 100x), Trypsin (2,5%); geneticin; trypan blue stain (0,4%)
ICN Biochemicals Inc.:	Ciprofloxacin hydrochloride
Merck:	Bromophenol blue; $CaCl_2$; DTT; EDTA; glycerol; KCl; NaCl; Na_2HPO_4; Tris base
Murex Diagnostics Ltd:	Streptolysin-O
Neolab:	Glycin
Riedel-de Haen:	APS
Roche:	Endoglycosidase H; Proteinase K
Serva:	HEPES
Sigma:	ATP-agarose (attachment through N-6); ATP Disodium salt Grad I; 5-bromo-2'-deoxyuridine (BrdU); BSA Fraction V; EGTA; Igepal CA-630; Leupeptin; Pepstatin A; Ponceau S solution; propidium iodide; TEMED; Tween 20

2.3 Kits

Amersham Biosciences:	ECL plus Western blotting detection system; HiTrap affinity columns NHS-activated
Applied Biosystems:	ABI PRISM™ Big Dye Terminators v 3.0 Cycle Sequencing Kit
Pierce:	BCA™ Protein Assay Kit
Qiagen:	QIAprep Spin miniprep Kit, QIAGEN Plasmid Midi Kit, QIAquick Gel Extraction Kit, QIAquick PCR Purification Kit,
Roche Diagnostics:	Expand High Fidelity PCR Kit

2.4 Solutions

LB-Medium (Luria-Bertani), pH 7,0
1% (w/v) bacto-tryptone
0,5% (w/v) bacto-yeast extract
1% (w/v) NaCl

PBS 10 x (phosphate buffered saline), pH 7,4
136mM NaCl
2,6mM KCl
1,8mM Na_2HPO_4 2 H_2O
1,5mM KH_2PO_4

Freezing medium
50% (v/v) DMEM
40% (v/v) FCS
10% (v/v) DMSO

FACS buffer
1x PBS
2,5% FCS

Solutions for DNA mini preparation:

P1, pH 8
50mM Tris base
11mM Na_2 EDTA
0,1mg/ml RNase A

P2
200mM NaOH
1% (w/v) SDS

P3, pH 5,5
3M KAc
pH was adjusted with glacial acetic acid

Solutions for agarose gels:

50x TAE (Tris-acetate-EDTA buffer)
200mM Tris-HCl
50mM EDTA, pH 8,0
5,71% (v/v) glacial acetic acid

6x TAE sample buffer
10% glycerol (v/v)
6x TAE
bromphenol blue

10x TBE
900mM Tris base
900mM boric Acid
20mM EDTA, pH 8,0

8x TBE sample buffer
5% (v/v) glycerol
5x TBE
bromphenol blue

Solutions for preparation of rVV and VV DNA:
2x HBS buffer (HEPES buffered saline), pH 7,14
50mM Hepes
20mM NaCl
1,5mM Na_2HPO_4

Buffer I, pH 8
20mM Tris base
10mM EDTA

3M NaAc, pH 4,8

4x Proteinase K buffer, pH 8
2,0% (w/v) SDS
40mM Tris base
20mM EDTA

*Solutions for antibody purification***:**
(all solutions were filtered through a 0,45µm filter prior to use)
Coupling buffer, pH 8,3
0,2M $NaHCO_3$
0,5M NaCl

Buffer A, pH 8,3
0,5M ethanolamine
0,5M NaCl

Buffer B, pH 4
0,1M acetate
0,5M NaCl

Buffer C, pH 8
50mM Tris base
0,5M NaCl

Buffer D, pH 9
50mM Tris base
0,5M NaCl

Start buffer
1x PBS

Elution buffer, pH 2,5
50mM glycine
1,15M NaCl

Solutions for SDS-PAGE and Western blotting:
RIPA lysis buffer (radio immune precipitation assay), pH 7,5
50mM Tris/HCl, pH 7,5
150mM NaCl
1,0% Igepal CA-630
0,1% (w/v) SDS
1% deoxycholate
Protease inhibitors (1mM PMSF, 10µM leupeptin, 1µM pepstatin A) were added before use.

5x Sample buffer
0,5M Tris/HCl pH 6,8
25% (v/v) glycerol
2% (w/v) SDS
2% (v/v) β-mercaptoethanol
bromophenol blue

Stacking gel
5% acrylamide (v/v)
65mM Tris/HCl pH 6,8
0,1% (w/v) SDS
14% (w/v) sucrose
TEMED
0,13% (w/v) APS

Separating gel
6-15% acrylamide (v/v)
43mM Tris/HCl, pH 8,8
0,1% SDS (w/v)
TEMED
0,12% APS (w/v)

10x Running buffer
252mM Tris base
1,92M glycine
1% (w/v) SDS

10x Blotting buffer
480mM Tris base
280mM glycin
20% (v/v) methanol was added before use

10x TBST (Tris buffer saline with tween)
0,1M Tris/HCl pH 8,0
1,5M NaCl
5% (v/v) Tween 20

Solutions for immunoprecipitation and EndoH digest.

Lysis buffer
140mM NaCl
5mM $MgCl_2$
20mM Tris/HCl, pH 7,6
Detergents (1% (w/v) Digitonin or 1% (v/v) Igepal CA-630) and protease inhibitors (1mM PMSF, 10µM leupeptin, 1µM pepstatin A) were added to buffer shortly before use.

Wash buffer B
150mM NaCl
10mM Tris/HCl, pH 7,6
2mM EDTA
Detergents were added to buffer shortly before use, 0,2% Digitonin (w/v) or 0,2% Igepal CA-630 (v/v)

Wash buffer C
500mM NaCl
10mM Tris/HCl, pH 7,6
2mM EDTA
Detergents were added to buffer shortly before use, 0,2% (w/v) Digitonin or 0,2% (v/v) Igepal CA-630

Wash buffer D
10mM Tris/HCl, pH 8,0

1x Sample buffer
80mM Tris/HCl, pH 6,8
5mM EDTA
34% sucrose (w/v)
bromophenol blue
Shortly before use 200µl 20% SDS (final concentration 3,2%) and 50µl 1M DTT (final concentration 40mM) were added to 1ml 1x sample buffer

EndoH buffer
50mM NaCitrate
0,02% (w/v) SDS
0,1% (v/v) Igepal CA-630
Shortly before use PMSF was added to a final concentration of 1mM.

4x Sample buffer
250mM Tris/HCl, pH 6,8
20mM EDTA
8% (w/v) SDS
60% (w/v) sucrose
bromophenol blue
Shortly before use 200µl 20%SDS (final concentration 11,2%) and 50µl 2M DTT (final concentration 80mM) were added to 1ml 4x sample buffer.

5x Running buffer

250mM Tris base

2M glycin

SDS was added to a final concentration of 0,1% only to the 1x Running buffer solution

Gel fixing

40% (v/v) methanol

10% (v/v) glacial acetic acid

Solutions for Peptide Translocation Assay:

TB, Translocation buffer

30mM KCl

5mM HEPES, pH 7,3

10mM NaCl

1mM $CaCl_2$

2mM EGTA

2mM $MgCl_2$

Lysis buffer

50mM Tris/HCl, pH 7,5

150mM NaCl

5mM $MgCl_2$

1% (v/v) Igepal CA-630

100mM ATP

For 10ml, ATP was dissolved in 5ml TB, then NaOH was added (~ 130µl, 10N) to reach pH 7 (tested with pH-stick). The ATP solution was filled up to 10ml and stored aliquoted at –20° C.

Solution for ATP binding assay:

ATP binding buffer

10mM Tris/HCl pH 7,0

150mM NaCl

5mM $MgCl_2$

Shortly before use PMSF, final concentration 1mM, IAA, final concentration 5mM, and 1% digitonin was added.

2.5 Oligonucleotides

Table 2.1 lists oligonucleotides applied for PCR analysis, sequencing analysis and for construction of gpUS6sol and the TAP1 chimera r8hVRT1.

TABLE 2.1
Oligonucleotides

Sequence 5'-> 3'	Application	Name
GGAGGCTGTGGCCTATGCAG	r8hVRT1	AH-h1/1
CTGCTTACAGCCCCTCTGACCACCAGCTGCCCACC	r8hVRT1	AH-h1/2
GCTGCTGACAGCCCCACTGGTCACCAGCTGCCCACC	r8hVRT1	AH-r1/2
GAATTCGCCGCCATGGATCTCTTGATTCGTCTC	gpUS6sol	-
CTAGCGAAACGCGTTCC	gpUS6sol	-
AACGATAATAGATACGGAACGGGA	PCR	aw-tk-11
CACACAGCAGTTAGTTTTACCACCA	PCR	aw-tk-12
GCACGGTAAGGAAGTAGAAT	seq	az-75-seq1
TTTTACCACCATTTCAGAT	seq	az-75-seq2
ACCGCCTTCGTTGTCAGTTATG	PCR/seq	AH-hTAP1-forward
CCTGTCTGGTTCTGTTGGAAAAAC	PCR/seq	AH-hTAP1-reverse
TCTCCTGCCTTGGGGAAATG	PCR/seq	AH-rTAP1-forward
TGTCACTAATGGACTCGCACACG	PCR/seq	AH-rTAP1- reverse
GGTGAACAACAAAGTCTTGATGTGG	PCR/seq	AH-hTAP2-forward
CCCAAAACTGCGAACGGTC	PCR/seq	AH-hTAP2- reverse
TGGCTGCTTGCTGGCTATGTAG	PCR/seq	AH-rTAP2-forward
CACCTTCTCGGCTGCTATTGTG	PCR/seq	AH-rTAP2- reverse

Mutations are shown in red

2.6 Antibodies

Antibodies used for Western blot (WB), immunoprecipitation (IP) and FACS methods are listed in Table 2.2.

TABLE 2.2
Antibodies

Antibody	Species	Specificity	Application	Reference
7510	rabbit p	gpUS6	WB, IP	Raised against aa 77-95 of gpUS6
E2 (7510)	rabbit p	gpUS6	IP	Purified 7510
1361	rabbit p	ICP47	IP	Raised against aa 69-88 of ICP47
7507	rabbit p	hum TAP1	WB, IP	Raised against aa 475-491 of hTAP1
148.3	mouse m	hum TAP1	WB, IP	(Meyer et al., 1994)
TAP1.28	mouse m	hum TAP1	IP	(Nijenhuis et al., 1996)
439.3	mouse m	hum TAP2	WB	(van Endert et al., 1994)
TAP2.17	mouse m	hum TAP2	IP	(Nijenhuis et al., 1996)
D90	rabbit p	rat TAP1	WB	(Momburg et al., 1992)
ratTAP1	goat p	rat TAP1	IP	(Antoniou et al., 2002)
D116	rabbit p	rat TAP2	WB	(Momburg et al., 1992)
ratTAP2	goat p	rat TAP2	IP	(Antoniou et al., 2002)
W6/32	mouse m	hum MHC	FACS	(Parham et al., 1979)
Secondary antibodies				
antibody	Species	Label	Application	Company
anti-rabbit	Goat	HRP	WB	Sigma
anti-mouse	Goat	HRP	WB	Dianova
anti-mouse	Goat	FITC	FACS	Dianova

p, polyclonal
m, monoclonal
HRP, horse radish peroxidase
FITC, fluorescein isothiocyanate

2.7 Cell lines

TABLE 2.3
Cell lines

Cell line	Source and characteristic	Reference
CV-1	African green monkey kidney fibroblast	ATCC: CCL-70
HU 143 tk^-	Tk-deficient human bone osteosarcoma	ATCC: CRL-8303
CMT64.5	TAP deficient mouse lung carcinoma	(Klar and Hammerling,
HeLa	Human epithelial cervix adenocarcinoma	ATCC: CCL-2
Rat-2	Rat embryonic fibroblast	ATCC: CRL-1764
L-M(Tk-)	Tk-deficient mouse fibroblast	ATCC:CCL-1.3
MRC-5	Human lung fibroblast	ATCC: X-55
Transfectants		
CMT64.5-*US6*	gpUS6 expressing CMT6.5	(Halenius et al., 2005)
HeLa-*US6*	gpUS6 expressing HeLa	(Hengel et al., 1997)
L-M(Tk-)-*US6*	gpUS6 expressing L-M(tk^-)	HH
HeLa-*ICP47*	ICP47 expressing HeLa	HH

HH, constructed by Hartmut Hengel

2.8 Viruses

In addition to the HCMV wild type lab strain AD169, recombinant vaccinia viruses (rVV) were used, a list of which is shown below. For construction of rVV's the Copenhagen wild type strain and the temperature sensitive VV mutant ts7 (Thompson et al., 1989) were applied.

TABLE 2.4
Recombinant vaccinia viruses

rVV	Ref	rVV	Ref
rVV-US6	(Hengel et al., 1997)	rVV-h4rT2	(Halenius et al., 2005)*
rVV-US6sol	EP	rVV-r4hT2	(Halenius et al., 2005)*
rVV-ICP47	(Banks et al., 1994)	rVV-h7rT2	(Halenius et al., 2005)*
rVV-hT1	(Russ et al., 1995)	rVV-r7hT2	(Halenius et al., 2005)*
rVV-hT2	(Russ et al., 1995)	rVV-r8hRVT1	EP
rVV-rT1	(Lobigs and Mullbacher,	rVV-rxhT1	EP
rVV-rT2	(Lobigs and Mullbacher,	rVV-WAT1	(Saveanu et al., 2001)*
rVV-FF1	M.L.	rVV-DelT1	(Saveanu et al., 2001)*
rVV-FF2	M.L.	rVV-RepT1	(Saveanu et al., 2001)*
rVV-h6rT1	(Halenius et al., 2005)*	rVV-WAT2	(Saveanu et al., 2001)*
rVV-r6hT1	(Halenius et al., 2005)*	rVV-DelT2	(Saveanu et al., 2001)*
rVV-h8rT1	(Halenius et al., 2005)*	rVV-RepT2	(Saveanu et al., 2001)*
rVV-r8hT1	(Halenius et al., 2005)*	rVV-UL118	(Atalay et al., 2002)

EP, described in Experimental procedures
M.L., gift from Dr. M. Lobigs (John Curtin School of Medical Research, Canberra, Australia)
*, Cloning of TAP subunits described elsewhere but constructed and analyzed as rVV within this thesis